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(12) (19) (CA) **Demande-Application**

(21) (A1) **2,270,320**
(22) 1999/04/28
(43) 1999/10/28

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(51) Int.Cl.[6] A61K 38/26, A61K 9/16, A61K 45/08
(30) 1998/04/28 (60/083/092) US

(54) **NOUVELLE COMPOSITION MICROSPHERE**
(54) **NOVEL MICROSPHERE COMPOSITION**

(57) A novel microsphere composition is described. The composition contains a biocompatible oil that increases the release of an active agent from the microspheres. In a preferred embodiment the active agent is GLP-1 and the microsphere composition can be used to treat diabetes.

BP File No. 2223-084/MG

ABSTRACT OF THE DISCLOSURE

A novel microsphere composition is described. The composition contains a biocompatible oil that increases the release of an active agent from the microspheres. In a preferred embodiment the active agent is GLP-1 and the microsphere composition can be used to treat diabetes.

WE CLAIM:

1. A microsphere composition comprising a biocompatible oil and a polymer capable of forming microspheres.

2. A composition according to claim 1 further comprising an active agent.

3. A composition according to claim 1 or 2 wherein the microspheres are composed of a biodegradable polymer.

4. A composition according to claim 3 wherein the polymer is poly-lactide-co-glycolide (PLGA).

5. A composition according to claim 4 wherein the polymer is PLGA-COOH.

6. A composition according to claim 1 or 2 wherein the polymer is selected from the group consisting of polyesters, polyketals, polyorthoesters, polyanhydrides, polyacetals, polyureas, polycarbonates, polyurethanes, polyamides and combinations thereof.

7. A composition according to any one of claims 1 to 6 wherein the biocompatible oil is olive oil.

8. A composition according to any one of claims 1 to 6 wherein the biocompatible oil is selected from the group consisting of olive oil, canola oil, soybean oil, sunflower oil, coconut oil, safflower oil, cotton seed oil, peppermint oil and chili pepper oil.

9. A composition according to claim 2 wherein the active agent is selected from the group consisting of hormones, proteins, peptides,

peptide analogs, peptide derivatives, drugs, vaccines, antigens vitamins, carbohydrates and lipids.

10. A microsphere composition according to claim 2 comprising: (a) a therapeutic peptide as the active agent; (b) olive oil as the biocompatible oil; and (c) a poly-lactide-co-glycolide as the polymer that forms the microspheres.

11. A composition according to claim 10 wherein the peptide is present in an amount from about 1% to about 20% of the total composition; the olive oil is present in an amount from about 20% to about 70% of the total composition; and the poly-lactide-co-glycolide microspheres are present in an amount from about 20% to about 79% of the total composition.

12. A composition according to claim 10 wherein the peptide is present in an amount of about 2% of the total composition; the olive oil is present in an amount of about 50% of the total composition; and the poly-lactide-co-glycolide microspheres are present in an amount of about 48% of the total composition.

13. A composition according to claim 12 wherein the therapeutic peptide is GLP-1.

14. A use of a composition according to any one of claims 2 to 13 to deliver an active agent to an animal.

15. A use of a composition according to any one of claims 2 to 13 to increase the bioactivity of an active agent in an animal.

16. A use of a composition according to any one of claims 1 to 13 to treat diabetes.

17. A use according to claim 16 wherein the microsphere composition is suitable for oral administration.

18. A method for preparing a microsphere composition comprising (a) adding a polymer capable of forming microspheres to a biocompatible oil; (b) adding an active agent in an aqueous solution to the polymer and oil solution; (c) pouring the mixture into a non-solvent solution under conditions which allow for the spontaneous formation of microspheres containing the naturally occurring oil and the active agent.

19. A method according to claim 18 wherein the polymer is dispersed in methylene chloride in step (a).

20. A method according to claim 18 or 19 wherein the non-solvent solution in step (c) is petroleum ether.

B&P File No. 2223-084/MG

Title: Novel Microsphere Composition

FIELD OF THE INVENTION

The present invention relates to a novel microsphere composition containing a biocompatible oil that increases the release of an active agent from the microspheres.

BACKGROUND OF THE INVENTION

Introduction

Therapeutic administration of peptide hormones has largely been restricted to parenteral routes due to the inherent capacity of the gastrointestinal tract to digest proteins. A number of different approaches have been developed that bypass the digestive system, thereby permitting systemic delivery of peptides and other therapeutic agents (1-7). The most conventional method of peptide delivery is by injections but more recently developed methods include continuous infusion pumps for hormones such as gonadotropin-releasing hormone and glucagon-like peptide-1 (GLP-1) (8,9), nasal sprays for peptides including antidiuretic hormone and calcitonin (3,4), mucosal dosage forms for insulin(10), and buccal tablets for GLP-1 (1). Although these are relatively successful techniques for peptide delivery, these approaches do have various limitations, including poor patient compliance (11,12), as well as the potential for tissue damage at the site of administration [eg lipodystrophy (13) and inhibition of nasal ciliary activity when delivered by nasal sprays (14)]. Alternative, less invasive routes of peptide delivery therefore continue to be a focus of investigation.

Recently, effective methods for encapsulating peptides and other drugs into orally-available, biocompatible microspheres have been developed (15-17). One important characteristic of these microspheres is that they can adhere to the intestinal mucosal epithelium (18), which slows their passage through the gastrointestinal system increasing their chance of being absorbed (15). In addition, microspheres, such as poly-lactide-co-glycolide (PLGA) and co-polymers of fumaric and sebacic acid (poly(FA:SA)), have been reported to undergo lymphoid-mediated uptake *via* Peyer's patches in the small intestine (15,19). Microspheres consisting of polyanhydride co-polymers of poly(FA:SA) have also been shown to

traverse across the small intestine through both para- and intracellular routes, after which at least some of the microspheres are found lodged in the liver and spleen (15).

Consistent with the ability of these microspheres to reach the systemic circulation, the oral bioavailability of dicumarol, an anti-coagulant, is increased 1.5- to 2-fold by encapsulation into poly(FA:SA) (15). A similar approach was also used for the successful oral delivery of the peptide hormone insulin (15). When insulin was encapsulated into these microspheres consisting of the polymers poly(fumaric acid) and PLGA [poly(FA:PLGA), 50:50] and then given orally to rats, a decreased glycemic response to an intraperitoneal glucose tolerance test was observed (15). However caution must be taken when giving insulin orally due to insulin capacity to cause hypoglycemia. In the case of insulin the exact amount a person receives is very important because to much insulin will result in hypoglycemia and too little will not prevent hyperglycemia. Therefore, although this new delivery system does show a lot potential for the treatment of diabetes and possibly other disorders, further studies are still required to control for the amount of insulin absorbed.

The insulinotropic peptide, Glucagon-like peptide-1 (GLP-1), has been proposed as a therapeutic agent for the treatment of type II diabetes (20,21) because of its ability to stimulate glucose-dependent insulin release (22,23) and possibly improve peripheral insulin sensitivity (24). GLP-1 has several advantages over insulin as for its potential for oral delivery, the most important of which is its apparent inability to cause hypoglycemia even at high peptide concentrations. However, there are two major problems associated with the administration of this peptide: (A) The peptide has a short *in vivo* half-life of 0.9 minutes (25), and (B) currently the major route of administering GLP-1 in humans is by parenteral administration (26). The active form of GLP-1 is a 31 amino acid hormone that is inactivated by the actions of dipeptidyl peptidase IV (DP IV) (25,27,28). To circumvent this inactivation the present inventors have utilized an analog of GLP-1, D-ala^2-GLP-1, that was designed to be

resistant to DP IV-mediated degradation. However, even with this modification, this peptide lost its biological activity within 4 hours of subcutaneous (*sc*) administration.

In view of the foregoing, there is a need in the art to provide improved oral delivery systems for the administration of biologicals such as peptides, especially GLP-1.

SUMMARY OF THE INVENTION

The present invention relates to a novel microsphere composition. In particular, the present inventors have demonstrated that microsphere compositions containing an active agent and a biocompatible oil show an increase in the release rate of the active agent as compared to a microsphere composition without the oil.

Broadly stated, the present invention provides a microsphere composition comprising a biocompatible oil and a polymer capable of forming microspheres.

In one embodiment the microsphere composition additionally includes an active agent. Accordingly, the present invention provides a microsphere composition comprising (a) an active agent and (b) a biocompatible oil encapsulated in (c) a microsphere formulation.

The present invention also provides a method of delivering an active agent to an animal comprising administering an effective amount of a microsphere composition according to the invention to an animal in need thereof.

The invention also includes a method of increasing the bioactivity of an active agent comprising administering a microsphere composition according to the present invention to an animal in need thereof.

The invention further includes a method of treating diabetes comprising administering a microsphere composition comprising GLP-1 to an animal in need thereof.

The present invention further provides a method for preparing a microsphere composition comprising (a) adding a polymer

capable of forming microspheres to a biocompatible oil; (b) adding an active agent to the polymer and oil solution; (c) pouring the mixture into a non-solvent solution under conditions which allow for the spontaneous formation of microspheres containing the biocompatible oil and the active agent.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figures 1A and B show the High Performance Liquid Chromatography (HPLC) analysis of native GLP-1 and D-ala^2-GLP-1 after incubation with DP-IV *in vitro*. **FIG. 1A** shows HPLC profiles of undigested and DP-IV digested native GLP-1 and D-ala^2-GLP-1 (after a 3 hour digest). **FIG. 1B** is a graph showing combined HPLC data for the change in elution position between digested vs. undigested native GLP-1 and D-ala^2-GLP-1 (*$P<0.05$). This data shows that our new analog is resistant to DP-IV degradation.

Figures 2A and B are graphs showing the effects of 10 ug of GLP-1 and 10 ug of D-ala^2-GLP-1 on the glycemic response to oral glucose in mice. **FIG. 2A** is a graph showing changes in glycemia in response to 1.5 mg glucose/g body weight (diamonds: PBS; squares, 10 ug native GLP-1; and triangles: 10 ug D-ala^2-GLP-1). **FIG. 2B** is a graph showing the area under the curve (AUC) for the glycemic responses. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ for D-ala^2-GLP-1 vs PBS; # $P<0.05$ for GLP-1 vs PBS; and +++ $P<0.001$ for D-ala^2-GLP-1 vs GLP-1. This data shows that the new analog of GLP-1 is more potent than native GLP-1.

Figure 3 is a bar graph showing percent of total peptide released from PLGA-COOH microspheres over a 9 hour incubation *in vitro*. Background #1 and #2: PLGA-COOH microspheres with olive oil only (n=1); 2% Glucagon: PLGA-COOH microspheres with glucagon (n=2); 18%alb/2%Gluc: PLGA microspheres with 18% albumin and 2% glucagon (n=2); 30%olv/2%Gluc: PLGA microspheres with 30% olive oil and 2% glucagon (n=1); 50%olv/2%D-ala^2: PLGA microspheres with 50% olive oil and 2% D-ala^2-GLP-1 (n=4); and 50%olv/2%Hex: PLGA microspheres with 50% olive oil and 2% hexenoyl-GLP-1 (n=2). These results show that we were able to develop a microsphere preparation that releases most of its peptide over a 9 hour period.

Figures 4A-C are graphs showing the release profile of PLGA-COOH-50% olive oil microspheres loaded with either 2% glucagon (n=2) (FIG. 4A), 2% D-ala^2-GLP-1 (n=4) (FIG. 4B) or 2% hexenoyl-GLP-1 (n=2) (FIG. 4C). Although these experiments were carried out over an 80 hour period, only the first 48 hours is shown as there was no further release of the peptide after this period. These results show that our microspheres release their peptide with an initial burst at t=1 hour followed by a gradual release of peptide with a peak at 7-9 hours.

Figure 5 is a transmission electron microscopy (TEM) analysis of the size of PLGA-COOH-microspheres. These experiments were done on microspheres without any peptide or olive oil. These results show that the average size of the microspheres was 0.96 m in size (n=3).

Figure 6 is a graph showing the analysis of the olive oil content in microspheres. **GLP-1 & OO** bar is microspheres containing 50% olive oil and 2% D-ala^2-GLP-1; **Olive Oil alone** bar is microspheres containing only 50% olive oil; **Polymer** bar represents empty microspheres that have no olive oil or peptide. The line represents the theoretical olive oil content of the microspheres. These results show that our microspheres have incorporated 60% of the added olive oil during their preparation.

Figure 7 is a graph showing the area under the curve of the delta blood glucose levels following repeated OGTT in normal CD1 mice at

0, 4 and 8 hours. Microspheres made of either 50% PLGA-COOH-50% olive oil (empty microspheres) or 48% PLGA-COOH-50% olive oil-2% peptide were given at t=0 hrs: **First Bar**, empty microspheres given *ip* (control#1; n=12); **Second Bar**, 5μg D-ala^2-GLP-1 given *sc* (n=6); **Third Bar**, *ip* injection of microspheres containing 2% D-ala^2-GLP-1 (equivalent to 50μg of peptide) (n=6); **Fourth Bar**, orally administered microspheres containing D-ala^2-GLP-1 (equivalent to 250μg of peptide) (n=9); **Fifth Bar**, 250μg of D-ala^2-GLP-1 given orally (control#2) (n=9); or **Sixth Bar**, 5μg of D-ala^2-GLP-1 given at t=0, 4 and 8 hrs prior to OGTT (control#3) (n=4). * $P<0.05$, *** $P<0.001$ vs. control#1. These results show that our microspheres deliver therapeutic levels of GLP-1 to non-diabetic mice.

Figure 8 is a graph showing basal blood glucose values at the beginning of each OGTT at t=0, 4 and 8 hours in diabetic db/db mice. **Squares**: control mice given no microspheres. **Open circles**: mice given 12.5 mg of D-ala^2-GLP-1-microspheres (* $P<0.05$, n=9). These results show that our microspheres lower basal blood glucose values over an 8 hour period in a model of type II diabetes.

Figures 9A-C are graphs showing basal blood glucose values in a mouse model of type II diabetes (*db/db* mice, which have a leptin receptor mutation). Delta blood glucose levels following repeated OGTT at 0 (FIG. 9A), 4 (FIG. 9B) and 8 (FIG. 9C) hours. Mice were treated at t=0 hrs with; nothing (controls, **diamonds**; n=9); or with orally administered microspheres containing 250μg D-ala^2-GLP-1 (**squares**; n=9). These results show that our microspheres cause a downward shift in the OGTT response in diabetic mice.

Figures 10A and B are graphical analysis of the results seen in figure 9 using area under the curve (AUC). **FIG. 10A:** Values expressed as the absolute area under the curve (area under the curve of the values including the basal blood glucose values). **FIG. 10B:** Values expressed as the delta area under the curve (area under the curve of the values independent of the basal blood glucose values). For figures **10A** and **10B**

the **black bars** are for control mice and the **grey bars** are for mice given microspheres containing D-ala^2-GLP-1. * $P<0.05$, *** $P<0.001$ vs. controls. These results show that our microspheres significantly lower the glycemic response to repeated OGTT in diabetic mice.

Figure 11 shows fluorescent staining of tissue after administration of microspheres containing Dextran-Texas Red. **Row A** tissue from the duodenum; **Row B** tissue from the ileum; **Row C** tissue from the spleen; and **Row D** tissue from the liver. **Column 1** tissues shows light microscopy representative pictures for each tissue (100X). **Column 2** shows the results from mice 2 hours after being fed 250µg of Dextran-Texas Red alone; **Column 3** shows the results from mice 2 hours after being fed Dextran-Texas Red-microspheres; and **Column 4** shows the results from mice 4 hours after being fed Dextran-Texas Red-microspheres. Areas of concentrated fluorescence were found in the duodenum, ileum, spleen and liver of Dextran-Texas Red-microsphere treated mice only, indicating that absorption of the microspheres occurred across the gastrointestinal tract.

DETAILED DESCRIPTION OF THE INVENTION

Microsphere Composition

The present invention relates to a novel microsphere composition. In particular, the present inventors have demonstrated that microsphere compositions containing an active agent and a biocompatible oil show an increase in the release rate of the active agent as compared to a microsphere composition without the oil. Consequently, the microsphere composition of the invention provides an improved delivery system for the administration of active agents. The bioactivity of the active agent is increased as compared to the bioactivity in the absence of the microsphere composition.

Broadly stated, the present invention provides a microsphere composition comprising a biocompatible oil and a polymer capable of forming microspheres.

In one embodiment the microsphere composition additionally includes an active agent. Accordingly, the present invention provides a microsphere composition comprising (a) an active agent and (b) a biocompatible oil encapsulated in (c) a microsphere formulation. The microsphere composition is preferably suitable for oral administration.

The microsphere can be composed of any biodegradable polymer that is capable of forming microspheres including, but not limited to, polyesters, polyketals, polyorthoesters, polyanhydrides, polyacetals, polyureas, polycarbonates, polyurethanes, polyamides and combinations thereof. The polymer is preferably in an amount from about 20% to about 79%, more preferably about 30% to about 50%, of the total composition. For oral preparations the polymer should be able to adhere to the intestinal mucosa, be taken up into the systemic circulation and be non-toxic. Preferably, the microspheres are formed from poly-lactide-co-glycolide (PLGA or PLAGA). More preferably, the polymer is poly-lactide-co-glycolide-COOH. The finding that PLGA-COOH microspheres containing an active agent and oil could increase the release of the active agent was surprising as PLGA-COOH microspheres containing active agent without oil are poor releasers of active agents.

The biocompatible oil may be any oil that is non-toxic and edible including, but not limited to, olive oil, canola oil, soybean oil, sunflower oil, coconut oil, safflower oil, cotton seed oil, peppermint oil or chili pepper oil. The oil is preferably present in a range from about 20 to about 70%, more preferably about 30% to about 50%, of the total composition. In one embodiment, the oil is olive oil.

The active agent may be any agent which one wishes to administer to a host including, but not limited to, hormones, proteins, peptides, peptide analogs, peptide derivatives, drugs, vaccines, antigens vitamins, carbohydrates or lipids. The active agent is preferably present in an amount from about 1 to about 20%, more preferably about 2 to about 10%, of the total composition. The active agent may optionally be dispersed in a solution (preferably an aqueous solution) prior to the

preparation of the microspheres. In one embodiment, the active agent is glucagon-like peptide-1 (GLP-1), more preferably D-ala^2-GLP-1.

In one embodiment of the invention, the microsphere composition comprises: (a) a therapeutic peptide and (b) olive oil in (c) a poly-lactide-co-glycolide microsphere formulation. In a specific embodiment, the microsphere composition comprises (a) a therapeutic peptide in an amount from about 1% to about 20%, preferably 2%, of the composition; (b) olive oil in an amount from about 20% to about 70%, preferably 50%, of the total composition in (c) a PLGA-COOH microsphere formulation in an amount from about 20% to about 79%, preferably 48%, of the total composition.

The present invention also provides a method for preparing a microsphere preparation comprising (a) adding a polymer capable of forming microspheres to a biocompatible oil; (b) adding an active agent to the polymer and oil solution; (c) pouring the mixture into a non-solvent solution under conditions which allow for the spontaneous formation of microspheres containing the biocompatible oil and the active agent. The active agent may optionally be dispersed in a solution (preferably an aqueous solution) prior to the preparation of the microspheres.

Preferably, the polymer is dispersed in methylene chloride prior to adding it to the oil. The active agent is preferably a peptide in an aqueous solution. The non-solvent solution is preferably petroleum ether.

Uses of the Microsphere Compositions

As hereinbefore mentioned, the microsphere compositions of the present invention show an increase in the release rate of the active agent as compared to microspheres compositions that do not contain a biocompatible oil. Accordingly, the present invention provides a use of a microsphere composition according to the invention to deliver an active agent. This aspect includes a method of delivering an active agent to an animal comprising administering an effective amount of a microsphere

composition according to the invention to an animal in need thereof. Preferably, the microsphere composition is administered orally.

The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired result.

The term "animal" as used herein includes all members of the animal kingdom, including humans.

The term "active agent" as used herein means any agent that one would like to administer to a host including all therapeutic, prophylactic and diagnostic agents. Examples of active agents include, but are not limited to, proteins, peptides, peptide analogs, peptide derivatives, hormones, vaccines, antigens, drugs, vitamins, carbohydrates or lipids.

The inventors have shown that delivering active agents such as therapeutic peptides using a microsphere composition of the present invention increases the bioactivity of the active agent as compared to when the active agent is administered without the microsphere composition. Accordingly, the present invention also provides use of a microsphere composition according to the invention to increase the bioactivity of an active agent. This aspect includes a method of increasing the bioactivity of an active agent comprising administering a microsphere composition according to the present invention to an animal in need thereof.

In a specific embodiment of the invention, the active agent is one which is useful as a therapeutic agent for the treatment of diabetes such as insulin or a GLP. In a preferred embodiment, the active agent is the peptide GLP-1. Accordingly, the present invention also provides a use of a microsphere composition of the invention to treat diabetes. This aspect includes a method of treating diabetes comprising administering a microsphere composition comprising GLP-1 to an animal in need thereof. The composition is preferably administered orally. The term "GLP-1" as used herein includes all analogs and derivatives of GLP-1 such as D-ala^2-GLP-1.

In particular, the present inventors have encapsulated an analog of GLP-1, D-ala^2-GLP-1 in microspheres made of 50% olive oil and 48% PLGA-COOH which were given to diabetic mice. The results, discussed in detail in the examples, demonstrate that the microspheres were effective in delivering therapeutic levels of GLP-1 over a ten hour period, thereby reducing both basal blood glucose levels and the glycemic response to repeated OGTT. As a result, the microspheres of the present invention are useful in the treatment of diabetes as patients could rely on a single dose of the microsphere composition to maintain therapeutic levels of GLP-1 throughout the day.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Materials and Methods

Petroleum ether and acetylnitrile were obtained from Fisher Scientific Ltd (Toronto, ON, Canada), gentamicin sulfate, methylene chloride, KOH, acidic boron triflouride, o-phosphoric acid and Triethylamine (TEA) from Sigma Chemicals (St. Louis, MO), Human placental Dipeptidylpeptidase IV (DP-IV) from Calbiochem-Novobiochem (La Jolla, CA), poly(DL-lactide-co-glycolide- COOH; 50/50) from Birmingham Polymers Inc. (PLGA-COOH, M_w ~11.5 kD; Birmingham, AL), glucagon from Eli Lilly Canada Inc (Toronto, ON, Canada), diprotin A from Calbiochem (San Diego, CA), GLP-1(7-36)amide from Bachem California Inc (Torrance, CA), and Dextran-Texas Red, 3000 MW, lysine fixable from Molecular Probes, Inc (Eugene, OR). D-Ala^2-GLP-1 and hexenoyl-His^1-GLP-1 were a kind gift from Dr. St. Pierre (UQAM, Montreal, QC, Canada). Spurr's Resin Kit was purchased from Marivac LTD (Halifax, NS), Cryomatrix from Shandon Inc. (Pittsburgh, PA), 3-O-Methyl-D-[1-3H] glucose from Amersham Life Science (Elk Grove, IL), and Olive Oil from Gallo (100% pure olive oil, from Unico Inc., Concord, ON). Triheptadecanoin from Nu-Chek Prep Inc. (Elysian, MN). EMLA Cream (2.5% Prilocaine and 2.5% lidocaine) and Xylocaine (5%, lidocaine

ointment, USP) from Astra Pharma Inc (Mississauga, ON), and Scintilation fluid (Ultima Gold MV) from Packard Instruments Inc. (Meriden, CT).

In vitro analysis of D-ala^2-GLP-1 Degradation by DP-IV

Incubation of 0.125 mU of DP-IV (specific activity = 5000 mU/mg protein) with either 33µg GLP-1(7-36)amide or 33µg D-ala^2-GLP-1 for 3, 8 or 24 hours was performed at 37°C in phosphate buffered saline (PBS). The reaction was quenched by adding 200µg of Diprotin A. The change in the elution position of the degradation product was compared to the undegraded peptide and to an internal standard of ^{125}I-GLP-1 using reversed-phase high-performance liquid chromatography (HPLC). The column was a µBondapak C_{18} column (3.9X300 mm) (Waters Associates., Milford, MA). The gradient ran from 45% to 85% of solution B (40% solution A and 60% acetonitrile; solution A: 0.1% o-phosphoric acid and 0.3% triethylamine) over 55 minutes.

Polymer preparation

Polymers were prepared using a modification of the microencapsulation by phase-inversion method described by Mathiowitz *et al* (15). In brief, 250µg of peptide and different amounts of albumin (depending on the percent loading desired) were dissolved in 25µl of *dd*H_2O in a glass tube. In a second glass tube, 0-10µl of olive oil (density 0.91 g/mL) was added (depending on the percent of the total weight required). In a third glass tube, a stock suspension of PLGA-COOH polymers was prepared (12.5 mg/ml in methylene chloride). 500µl of the PLGA-COOH solution was then added to the glass tube containing the olive oil, vortexed, and added to the 25µl *dd*H_2O. This was vortexed for 5 seconds and then rapidly poured into 50 mL of unstirred petroleum ether (methylene chloride:petroleum ether ratio of 1:100), resulting in the spontaneous formation of PLGA-COOH microspheres. This was allowed

to air-dry in a fumehood for approximately 3 hours and then the microspheres were then harvested.

Size Analysis

Transmission Electron Microscopy (TEM): Microspheres were prepared with no peptide or olive oil and then infiltrated with Spurr's Resin and cured for 2 hours at 65°C. 5-10µm sections were then prepared and observed at 75 kV.

Particle size determination by Dynamic Light Scattering (DLS): A Nicomp 370/Autodilute Submicron Particle Sizer (Pacific Scientific, Instruments Div., Silver Springs, MD) was used for the analysis of 1 mg of probe sonicated or unsonicated D-ala^2-GLP-1-Olive Oil-PLGA-COOH microspheres. The light source in the Nicomp was a HeNe laser beam at 632.8 nm, and the angle of detection was 90°. All analysis were done using a Gaussian analysis method.

Analysis of Oil Content

Teflon-silanized coated glass tubes were used to prevent the fatty acids from sticking to the tubes. Three samples of olive oil alone or 10 mg of PLGA-COOH microspheres with no olive oil, with olive oil or with olive oil and D-ala^2-GLP-1 were analyzed using this method. The triglycerides were broken down using 1 M KOH in methanol under N_2 gas at 90°C for 2 hours. BF_3 (acidic boron trifluoride in methanol) was then used as a catalyst for methylation of the FFA to give FFA-methyl esters. A Hewlett Packard 5890A Gas Chromatograph was used to separate the different types free fatty acids (FFA). To aid in the determination of the amounts of each type of FFA in olive oil an internal standard of triheptadecanoin was added to each sample. Using the amounts of FFA in each sample and knowing the composition of olive oil, the amount of olive oil in the microspheres was determined in mg of olive oil/mg of microspheres (note olive oil alone was analyzed in varying concentration (3,5 and 7µl of olive oil) to produce a standard curve for comparison).

In vitro analysis of Peptide Release

A known amount of polymer was weighed into a polypropylene tube and 10 ml of PBS (pH 7.4) with 0.05 mg/ml of gentamicin sulfate. The tube was shaken vigorously for 20 seconds and then centrifuged for 5 minutes at 1300Xg. A sample of the PBS-Gentamicin was collected, then the tube was manually shaken for 20 seconds to resuspend the polymer, after which the suspension was incubated at 37°C. Additional samples were taken every hour for the first 9 hours and then after 24, 32, 48, 56, 72 and 80 hours.

Oral Glucose Tolerance Tests (OGTT)

Female CD1 mice (6-8 weeks old) obtained from Charles River Canada (Montreal, QC, Canada), or female db/+ or db/db mice (7-11 weeks old) obtained from Jackson Laboratories (Bar Harbor, ME, USA) were used for all experiments. Mice were housed under a light/dark cycle of 12 hours, and were fasted for 16-17 hours prior to the day of experimentation. All experiments were initiated between 0900 and 1000. Mice were given 1.5 mg of glucose per gram of body weight orally through a gastric gavage tube (18 or 22 gauge gavage needle) and blood was collected from a tail vein at 0, 10, 20, 30, 60, 90, 120 minutes. Blood glucose levels were measured with a One Touch Glucose meter (Lifescan Canada LTD, Burnaby, BC, Canada). At t=0 minutes either; polymers were administered orally with the glucose using a 22G gavage needle, or intraperitoneally (*ip*) using a 25G5/8 needle (note: some of the polymer preparations were probe-sonicated for 4-8 seconds at a 16 kc/s with a peak-to-peak amplitude of 5-7 m, to allow for smooth delivery), or 5μg of D-ala^2-GLP-1 was delivered by subcutaneous (*sc*) or *ip* injection with 25G5/8 needle. At the end of each oral glucose tolerance test (OGTT), EMLA Cream or Xylocaine was applied to the tip of the tail. In some mice, OGTT's were repeated at t=4 and 8 hours.

Determination of Gastric Emptying

Gastric emptying measurements were done in CD1 mice along with an OGTT using 3-O-Methyl-D-[1-3H] glucose similar to a

method described by Gedulin and Young (29). The protocol is the same as above for the OGTT except 5µCi of 3-O-Methyl-D-[1-3H] glucose was added to the glucose solution given to the mice. Plasma blood samples at 0, 10, 20, 30, 60, 90 and 120 minutes were taken to measure cpm. 3-O-Methyl-D-[1-3H] glucose has been shown to be a good measure of gastric emptying (29). Thus, after determination of the blood glucose concentrations, an additional 4-5 drops of blood was obtained from the mice and placed into a microhematocrit capillary tube, sealed at the end with critseal and then centrifuged to collect 5µl of plasma. Which was added to plasma scintilation fluid and counted on a -counter.

Route of Absorption and Targets Organs of the Microspheres

Dextran-Texas Red was encapsulated into PLGA-COOH microspheres with olive oil as describe above for the encapsulation of peptides. Dextran-Texas Red, 3000 MW, was chosen because it had a similar MW to that of GLP-1. These microspheres were then given to CD1 mice by oral gavage. Tissues (stomach, duodenum, ileum, large intestine, cecum, kidney, liver and spleen) were collected at 2 or 4 hours after administration and frozen at -70°C in cryomatrix. After 12-14 hours, fresh unstained tissue sections of 15-20µm in size were prepared using a cryostat. Tissue section were viewed immediately after cutting using a confocal microscope with tungsten mercury lamp (filter wave length at 595 nm).

Radioimmunoassays (RIAs)

RIAs for immunoreactive glucagon (IRG) and immunoreactive GLP-1(7-36)amide were carried out (30) using antiserum 04A (Dr. R.H. Unger, Dallas, TX), which cross-reacts with the free C-terminal end of glucagon, and antiserum GLP-1(7-36)amide (Affinity Research, Nottingham, UK), which detects C-terminally amidated forms of GLP-1, respectively. Plasma was prepared for GLP-1 RIA by reversed-phase extraction on a C18 SepPak (Waters Associates, Milford, MA) and extracts were dried *in vacuo* prior to assay (30,31).

Statistics

Statistical significance was assessed by ANOVA using n-1 custom hypotheses tests, or by Tukey's studentized range test, as appropriate, using a Statistical Analysis System program (SAS Institute, Cary, NC). All data are expressed as the meanSEM.

RESULTS

Example 1: Increasing GLP-1 Duration of Action

(A) *In vitro:*

Reversed-phase high-performance liquid chromatography (HPLC) was used to compare the degradation of D-ala^2-GLP-1 to that of native GLP-1 by DP-IV *in vitro*. HPLC analysis of D-ala^2-GLP-1 showed no DP-IV cleavage over a 24 hour period whereas native GLP-1 was cleaved within 3 hours when incubated with DP-IV *in vitro* ($P<0.05$, n=3). (The results are shown in Figure 1.)

(B) *In vivo:*

To determine whether D-ala^2-GLP-1 maintained its biological activities *in vivo*, mice were given a *sc* injection of saline or 10μg of native GLP-1 or D-ala^2-GLP-1, and their response to an oral glucose tolerance test (OGTT) was determined. Native GLP-1 significantly reduced the glycemic area under the curve over a period of 2 hours (AUC) in comparison to saline (from 337±80 to 209±29 mM*120 minutes; $P<0.05$, n=6), and D-ala^2-GLP-1 further reduced the AUC significantly in comparison to both saline and native GLP-1 (-552 mM*120 minutes; $P<0.001$, n=6). (The results are shown in Figure 2.) Thus, this new analog of GLP-1 exhibits enhanced biological activity *in vivo* as compared to native GLP-1, likely because of its ability to resist DP-IV inactivation.

Example 2

(2A) Designing an Oral Delivery System for D-ala^2-GLP-1

The preferred release pattern for the delivery of GLP-1 to type II diabetics would be over a 9-12 hour period because patients could rely on a single dose to maintain therapeutic levels of GLP-1 throughout the day. PLGA-COOH is an excellent polymer for the formation of microspheres

because it adheres to the intestinal mucosa of the gastrointestinal tract due to its -COOH end groups (15,32-34). PLGA has also been shown to be taken up taken up into the systemic circulation (15,19), and its degradation products are non-toxic (16). However, the degradation rate of PLGA is very slow, and it is therefore not considered to be useful in the delivery of therapeutic levels of compounds (35). Consistent with these findings, initial studies using PLGA-COOH microspheres alone showed that the release of encapsulated glucagon was near background levels over 9 hours (n=2; Figure 3), and did not increase even after 408 hours of incubation (data not shown).

In an attempt to increase the rate of release of peptide from PLGA-COOH microspheres, we tested the effects of incorporating albumin. Using a range of concentrations to obtain up to 60% percent of the total weight of the microsphere with albumin, the best release obtained was only 9.8% of the total glucagon (250µg of peptide/ 12.5 mg of microspheres) encapsulated within the microspheres over a 9 hour period (Figure 3, only 18% albumin shown; n=2). In a separate series of studies, olive oil was also tested for effects on the release of peptide from PLGA-COOH microspheres. Olive oil is non-toxic, and is soluble in methylene chloride, but not in petroleum ether, all of which are important considerations for the preparation of our microspheres. The addition of olive oil consisting of 30% to 50% percent of the total weight of the microspheres increased the release of encapsulated glucagon to 43.1% (n=1) and 68.4% (n=2) of total peptide over a 9 hour period, respectively (Figure 3).

The 50% olive oil-PLGA-COOH microsphere preparation was selected for further studies using the D-ala^2-GLP-1 and hexenoyl-His^1-GLP-1 analogs of GLP-1. In preliminary studies, we determined that this new analog, D-ala^2-GLP-1, is detected in our GLP-1 assay in a similar fashion to that of wild-type GLP-1 (data not shown). When encapsulated within PLGA-COOH microspheres containing 50% olive oil, the release of D-ala^2-GLP-1 and hexenoyl-His^1-GLP-1 reached 10421% (n=4) and 89.2% (n=2) of the total peptide within 9 hours,

respectively (Figure 3). The release profiles for PLGA-COOH microspheres containing olive oil and either glucagon (n=2) or D-ala^2-GLP-1 (n=4) demonstrated an initial burst phase at about t=1 hour, followed by a decline (likely due to loss of peptide *via* adsorption to the test tube and to the microsphere surface), a second release phase with a peak at t=7-9 hours, and then a further decline over the ensuing 48 hours (Figure 4). A similar profile was observed for hexenoyl-His^1-GLP-1, with the only apparent difference being the absence of the 'decline phases'. We decided that the microspheres consisting of 50% olive oil- 48% PLGA-COOH- 2% peptide were the preferred composition for *in vivo* studies.

Each of the peptides were likely encapsulated within these 50% olive oil-PLGA-COOH microsphere preparation because if you encapsulate a water soluble dye (Dextran-Texas Red; purple color) into these microspheres and then place these microspheres into water, there was no or very likely change in the color of the water after the microspheres had settled to the bottom of the tube. These results were consistent even after the microspheres were probe sonicated for 8 seconds.

(2B) Size of the microspheres

Two methods were used to determine the size of the microspheres, both of which gave similar results. The first method was transmission electron microscopy (TEM). These experiments were carried out with microspheres without any peptide or olive oil, giving a result of 0.96μm in size (Figure 5; n=3). We also used a Dynamic Light Scattering (DLS) method for determining particle size (36,37). Using microspheres containing olive oil and D-ala^2-GLP-1, which were either sonicated or unsonicated (Table 1; n=4). The size of the microspheres were 1.01μm for the sonicated and 2.32 m for the unsonicated. These results suggested to us that, because our microspheres were <5μm in size, they are a suitable size for absorption across the gastrointestinal tract (38).

(2C) Olive Oil Content of the Microspheres

These experiments were performed on microspheres made of PLGA-COOH with no peptide or olive oil; with olive oil or with olive oil

and peptide. Microspheres made of PLGA-COOH alone had no measurable amount of olive oil; those made of PLGA-COOH and olive oil had 0.22±0.07 mg of olive oil/ mg of microsphere and those made of PLGA-COOH with olive oil and D-ala^2-GLP-1 had 0.30±0.02 mg of olive oil/ mg of microsphere (figure 6) as compared to the 0.50 mg of olive oil/ mg of microsphere that was added during the microsphere preparation. This data suggests that about 60% of the olive oil added to the preparation was incorporated into the microspheres.

Example 3

(3A) Effects of D-ala^2-GLP-1-microspheres *in vivo*

When 2.5 mg D-ala^2-GLP-1-microspheres (equivalent to 50µg of peptide at 2% loading) were injected *ip* into non-diabetic CD1 mice followed by repeated OGTT at 0, 4 and 8 hours, the glycemic area under the curve (AUC) in response to the repeated OGTT was significantly reduced as compared to controls at 0, 4 and 8 hours (from 346±53 to 93±59 mM*120 minutes, P<0.001 at 0 hours; from 424±24 to 219±50 mM*120 minutes, P<0.001 at 4 hours; from 461±29 to 282±49 mM*120 minutes, P<0.001 at 8 hours; n=12 and 6 respectively) (figure 7). When 5µg of D-ala^2-GLP-1 was given alone by *ip* injection at t=0 hours, there was a significant difference as compared to controls only at t=0 hours but not at any of the other time points (from 346±53 to 161±67 mM*120 minutes, P<0.001 at 0 hours; n=6). This suggested to us that the peptide alone lost its ability to affect the glycemic response within 4 hours and that when the microspheres are given by an *ip* injection, bioactive GLP-1 levels are maintained over an 10 hour period.

When 12.5 mg D-ala^2-GLP-1-microspheres (2% loading; equivalent to 250µg of peptide) were given orally to mice (non-diabetic CD1 mice) (at t=0 hours) followed by repeated OGTT at 0, 4 and 8 hours, the glycemic AUC was significantly reduced as compared to controls at the 4 and 8 hour time points (from 424±24 to 247±50 mM *120 minutes, P<0.001 at 4 hours; and from 461±29 to 371±53 mM *120 minutes, P<0.05 at 8 hours; n=9) (figure 7). This demonstrated that our microsphere preparation was

effective in delivering bioactive levels of GLP-1 orally to mice over a 10 hour period.

In an attempt to control for the amount of peptide given within the microspheres, 250µg of D-ala^2-GLP-1 was given orally to mice. The glycemic AUC was not significantly affected as compared to controls at any of the time points studied (295±54 mM *120 minutes, at 0 hours; 340±28 mM *120 minutes, at 4 hours; 514±38 mM *120 minutes, at 8 hours; n=9) (figure 7). Another control to predict the approximate amount of peptide present at each time point was carried out by giving an *ip* injection of 5µg of D-ala^2-GLP-1 just before each OGTT (one injection at each of t=0, 4 and 8 hours). The glycemic AUC was significantly reduced as compared to controls at all of the time points studied (from 346±53 to 198±56 mM*120 minutes, $P<0.001$ at 0 hours; from 424±24 to 128±37 mM*120 minutes, $P<0.001$ at 4 hours; from 461±29 to 170±32 mM*120 minutes, $P<0.001$ at 8 hours; n=4) (figure 7). These results are therefore similar to those found for the administration of 12.5 mg of D-ala^2-GLP-1-microspheres (containing 250µg of peptide), suggesting that approximately 1-2% of the peptide was available at the 4 and 8 hour OGTT.

(3B) Effects of D-ala^2-GLP-1-microspheres on Gastric emptying

High concentrations of GLP-1 have been reported to affect gastric emptying in humans (39,40) as well as in rodents (41). Therefore to test whether this is the case with the D-ala^2-GLP-1-microspheres, we added 5µCi 3-O-Methyl-D-[1-3H] glucose to the glucose given to control CD1 mice, or mice given an *ip* injection of 5µg of D-ala^2-GLP-1, oral microspheres or the mice given 250µg of oral D-ala^2-GLP-1, for OGTT at either t=0, 4 or 8 hours. The results showed that there was no significant effect of the microspheres, or any other treatment, on gastric emptying in these mice at all time points (data not shown).

(3C) Effects of D-ala^2-GLP-1-microspheres in a Model of Type Diabetes

When D-ala^2-GLP-1-microspheres were given orally to diabetic *db/db* mice (at 0 hours), followed by repeated OGTT at 0, 4 and 8

hours, the basal blood glucose values were reduced at 4 hours (from 13±1.4 to 10±1.4 mM) and significantly reduced at 8 hours (from 12±1.1 to 80±.9 mM, $P<0.05$, n=6) in comparison to controls, *db/db* mice given no microspheres (figure 8). The OGTT response at t=0 hours for both mice treated with the D-ala^2-GLP-1-microspheres and the controls were not distinguishable from each other but at t=4 and 8 hours the OGTT curves were drastically different from each other with the diabetic mice receiving D-ala^2-GLP-1-microspheres curve being shifted downward (figure 9). In addition, the absolute glycemic AUC (AUC calculated including basal blood glucose values) was reduced significantly as compared to controls at 4 and 8 hours time points (from 2589±105 to 1921±138 mM*120 min, at 4 hour, $P<0.001$; and from 2460±152 to 1835±88 mM*120 min, at 8 hour, $P<0.001$; n=6) (figure 10A). Also, the delta glycemic AUC (AUC independent of the initial basal blood glucose value) was also significantly reduced in comparison to controls at 4 hour time point (from 1000±140 to 714±49 mM*120 min, $P<0.05$; n=6) (figure 10B). These findings demonstrate that, in diabetic mice, D-ala^2-GLP-1 encapsulated into PLGA-COOH-microspheres is effective in delivering therapeutic levels of GLP-1 orally over an 10 hour period, thereby reducing both basal and the glycemic response to repeated OGTT.

Example 4: Route of Absorption and Targets Organs of the Microspheres

Dextran-Texas Red was encapsulated into PLGA-COOH microspheres with olive oil and then given orally to CD1 mice. No fluorescence was seen in mice treated with microspheres containing no Dextran-Texas Red. Diffuse fluorescence was found in the duodenum, ileum, liver, kidney and the spleen of the mice given 250µg of Dextran-Texas Red alone orally and in mice given microspheres containing Dextran-Texas Red 2 and 4 hours after administration. However, there was significantly more intense fluorescence seen in the microspheres treated mice as compared to the 250µg of Dextran-Texas Red alone (figure 11). These results suggested that, although diffuse fluorescence was seen in both Dextran-Texas Red-microspheres and in

mice treated with Dextran-Texas Red alone, the concentrated fluorescence seen in the microsphere treated mice was likely a result of the absorption of the microspheres in the duodenum and ileum after which ending up in the liver, kidney and spleen.

DISCUSSION

The results of the present study indicate that encapsulation of GLP-1 in microspheres composed of 48% PLGA-COOH, 50% olive oil and 2% peptide permits delivery of bioactive GLP-1 through the oral route in mice.

The major contributor to the relatively short half-life of 0.9 minutes for GLP-1 (25) is the enzyme Dipeptidyl-Peptidase-IV (DP-IV), which cleaves the first two amino acids (His^1-ala^2) from the N-terminus of GLP-1 and thus inactivating the peptide (27,46). To overcome this short half-life, Dr. Serge St. Pierre from the University of Quebec in Montreal, synthesized an analog of GLP-1 that was designed to be DP-IV resistant. We have shown that this novel GLP-1 analog, D-ala^2-GLP-1, is resistant to DP-IV cleavage over a 24 hour period *in vitro* and maintains its biological activities *in vivo*. These data suggest that this peptide can potentially be used in the treatment of type II diabetes. However, even with this resistance to DP-IV cleavage the biological activities of D-ala^2-GLP-1 were lost 4 hours after an *ip* administration (figure 7), thus still necessitating the need for repeated injections of the peptide if it is to be used as a treatment of type II diabetes.

Therefore because of possible non-compliance with a regimen of repeated GLP-1 injections, we have designed a novel oral delivery system for this peptide. Although a number of investigators have utilized PLGA and related polymers for oral peptide delivery (11,15,19), several novel aspects of the present study warrant discussion. First, no previous studies have reported on the use of PLGA-COOH as the base polymer for microsphere preparation. The modification of adding a -COOH end group to these polymers was done to decrease the passage of microspheres through the gastrointestinal tract and thus increases there chance of being

absorbed (32-34). However, as we and others (47,48) have demonstrated in our *in vitro* studies, the use of PLGA or PLGA-COOH alone did not permit release of the test peptide within a time frame that is reasonable for peptide therapeutics.

Thus, in an attempt to increase the rate of release of peptide from these microspheres, the PLGA-COOH was combined with other more soluble biocompatible compounds, including albumin and olive oil. Albumin had only a marginal effect on increasing the rate of release of peptide. The addition of increasing concentrations of olive oil on the other hand, up to 50% of the total weight of the microspheres, clearly increased the ability of peptide to be released from the microspheres, such that approximately 70-100% of the peptide was consistently recovered within 9 hours of incubation *in vitro*, regardless of the peptide being tested. Interestingly, despite the excellent recovery of peptide during these incubations, microspheres were still clearly visible, even after 17 days of incubation. These findings suggest that the major mechanism of release of peptide from these microspheres is diffusion, rather than through degradation and/or bulk erosion, as reported for other polymer preparations (49,50).

As no other studies had previously reported on the use of olive oil in their polymer preparation or any other commercially available ingestable oils for that matter, these novel findings clearly require further testing. There are two studies to date that have used oleic acid, a major constituent of olive oil (70%), to affect polymer function. One study attempted to use oleic acid to enhance the uptake of polystyrene microparticles into lymph of rats (51). In this study they found that microparticles in a solution of 6% oleic acid did not enhance the absorption of the polystyrene microparticles and in fact decreased their absorption as compared to saline in a closed intestinal-loop model. What is interesting is that lecithin (another lipid delivery vehicle) did marginally enhance the absorption of the polystyrene microparticles in this model suggesting that this lipid could also be used as a substitute for

olive oil in our experiments. In another study they used oleic acid, and other low molecular weight compounds, to enhance the degradation rate of poly(-caprolactone) and PLGA (47) by applying it to the surface of the polymer and then measured the degradation products. They found that the rate of PLGA hydrolysis was increased when oleic acid was applied to the surface of the polymer, suggesting a change in the intrinsic reactivity of the glycolate linkage in PLGA.

What is unique about our experiments in comparison to these studies using oleic acid is that we have used olive oil as an integral component of our microspheres as opposed to a mode of delivery or as a topical application. Support for olive oil being part of our microspheres comes from our studies that our microspheres have olive oil incorporated into the microspheres using gas chromatography. Also, in view of the study (47) showing that oleic acid increases the rate of degradation of PLGA, it is likely that the addition of olive oil increased the rate of degradation of PLGA-COOH-microspheres, from the inside out, thus allowing for an increase in the release of peptide such that there was a nearly complete recovery of the total amount of peptide added (as seen in our *in vitro* release studies).

In general, microspheres that are <5μm in size have been suggested to be suitable for absorption across the gastrointestinal tract (19,38,52,53). There are four proposed sites of absorption of microspheres which include the villus tips, intestinal macrophages, enterocytes and Peyer's patches (38). The mechanism of absorption at each of these sites dictates the size of particles that can be absorbed. The proposed mechanism of absorption across villus tips is persorption which would allow for particles that are 5-150μm in size to be absorbed, intestinal macrophages can absorb 1μm particles by phagocytosis, enterocytes can absorb <200 nm particles by endocytosis and Peyer's patches can absorb <10μm particles by transparacellular means. Although all of these sites likely play role in absorption, the major site of absorption of all

microparticles has been suggested to be the Peyer's patches (54,55). Along these lines, the main site of PLGA microspheres absorption in the size range of 1-5μm has been shown to be the Peyer's patches (19). After absorption of the PLGA-microspheres they have been found in the lymph nodes, spleen, kidneys and liver. The microspheres that we produced were about 1μm in size, suggesting to us that they were a suitable size for absorption across the gastrointestinal tract. In addition, the sites where they were found included the duodenum, ileum, spleen, kidney and liver at 2 and 4 hours after administration, as determined by encapsulating dextran-Texas Red into our microspheres.

In vivo studies with D-ala^2-GLP-1-microspheres clearly indicated that the peptide was release into the circulation of the mice and retained its biological activity when delivered through the *ip* and oral routes of administration. An *ip* injection of D-ala^2-GLP-1-microspheres permitted the delivery of bioactive levels of peptide that results in a significant reduction of the area under the curve in response to OGTT at t=0, 4 and 8 hours. The time course of action for these effects differed markedly from that of non-encapsulated peptide, such that a much more prolonged duration of action was observed for the D-ala^2-GLP-1-microspheres (for a least 10 hours). The effectiveness of this peptide over the entire 10 hour period tested is ideal for the delivery of a therapeutic peptide that is required over a prolonged period of time.

When we gave our D-ala^2-GLP-1 alone to non-diabetic mice we found that the duration of action of a single dose of *sc/ip* D-ala^2-GLP-1 is extremely short (<4 hours), orally administrated D-ala^2-GLP-1 (250μg) is biologically inactive. When the D-ala^2-GLP-1-microspheres were given orally it permitted the delivery of bioactive peptide that resulted in a significant reduction of the area under the curve in response to OGTT at t=4 and 8 hours but not at t=0 hours. If the major site of microsphere absorption is by the Peyer's patches then for an effect to be seen one must wait until the microsphere get to the ileum where the majority of the Peyer's patches are located (56-61). The amount of biologically active

peptide at t=4 hours in mice treated with oral microspheres containing a total of 250μg of D-ala^2-GLP-1 was approximately 5μg of peptide (determined by comparing the response of oral microspheres at t=4 hours to a 5μg *ip* injection given at t=4 hours). Therefore, in these mice our microsphere preparation permits oral delivery of therapeutic levels of peptide over an 10 hour period equivalent to repeated injections of 5μg of peptide (1-2% of the total peptide). This low bioavailability may be due to a slow continuous release mechanism. It is possible that 1% of the total peptide absorbed is released per hour. This is assuming though, that 100% of the microspheres are absorbed. But this is not likely the case because it has been shown previously that only about 12% of PLGA microspheres in the 1-5mμ size range are absorbed across the gastrointestinal tract (19).

D-ala^2-GLP-1-microspheres were also given to a mouse model of type II diabetes to determine whether or not therapeutic levels of GLP-1 can be delivered orally in diabetes. The mouse model we chose to use was the *db/db* mouse which have a leptin receptor mutation that inactivates the receptor. This mutation results in the mice being hyperphagic, hyperglycemic, hyperinsulinemic and obese (62-64). The late onset of hyperglycemia and hyperinsulinemia in these mice is a similar characteristic found in type II diabetes (63). We found that there was no difference seen between control mice and mice treated with D-ala^2-GLP-1-microspheres at t=0 hrs, as expected, however, the response to orally administered microspheres at t=4 and 8 hours was a downward shift in both basal and stimulated glycemia as compared to control mice. Therefore in addition to being able to deliver orally D-ala^2-GLP-1 to non-diabetic mice, we are also able to orally delivery therapeutic levels of peptide over a 10 hour period in a diabetic model of type II diabetes. These results suggest that this technique for orally delivery of peptides may be useful in the treatment of type II diabetes and as well as other disorders requiring the continuous presence of peptide.

In conclusion, the results of the present study demonstrate that oral delivery of therapeutic peptides can be accomplished through a novel approach to encapsulate peptides within biocompatible microspheres containing a biocompatible oil. The approach is clearly feasible for delivery of a wide variety of bioactive peptides and possible other compounds. Furthermore, one skilled in the art will appreciate that the rate of peptide release could be modified by altering the percent composition of the oil in the microspheres. Also, additional types of ingestable oils may be used to further adjust the polymer preparation to increase the absorption of the microspheres. Another possibility may be to conjugate molecules to our microspheres that can actively work to increase the absorption of the microsphere preparation. Therefore, with the ability to modify the release kinetics as well as with some minor modification to improve the total amount of microspheres absorbed, this method will be extremely effective in orally delivering therapeutic levels of active agents such as peptides.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

TABLE 1

Preparation	Average Size (um)	Standard Deviation (um)
Sonicated Polymers	1.00	1.02
Non-Sonicated Polymers	2.30	2.06

Analysis of the size of the microspheres containing D-ala^2-GLP-1 using a Dynamic Light Scattering (DLS) method for determining particle size. The microspheres used for these experiments contained olive oil and D-ala^2-GLP-1 and were either sonicated or unsonicated (n=4). Analysis was performed using Gaussian analysis. These results show that our sonicated microspheres are about 1μm in size, therefore these were used in all subsequent studies *in vivo*.

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FIG. 1A

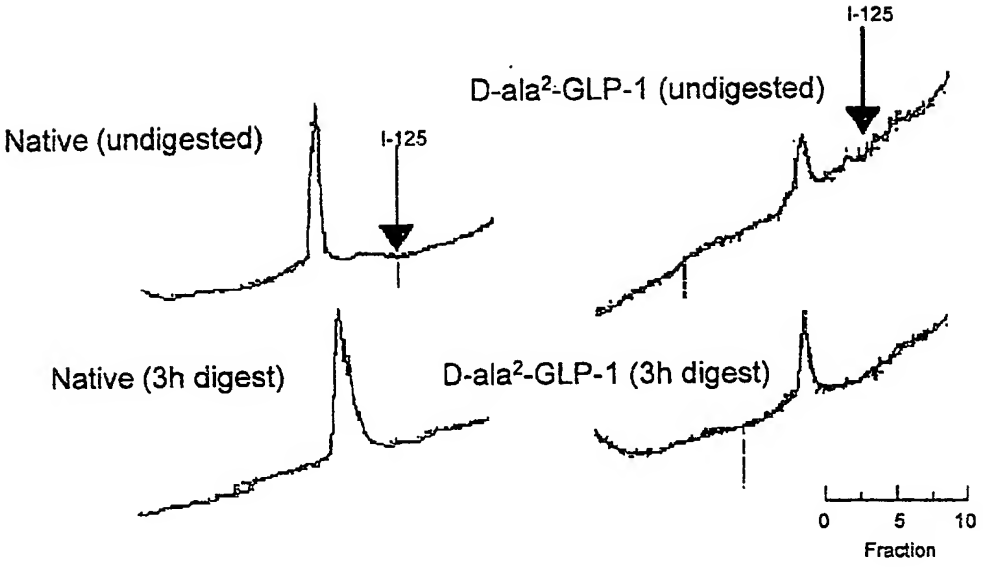
I-125
D-ala²-GLP-1 (undigested)
Native (undigested)
I-125
Native (3h digest)
D-ala²-GLP-1 (3h digest)
0
5
10
Fraction

FIG. 1B

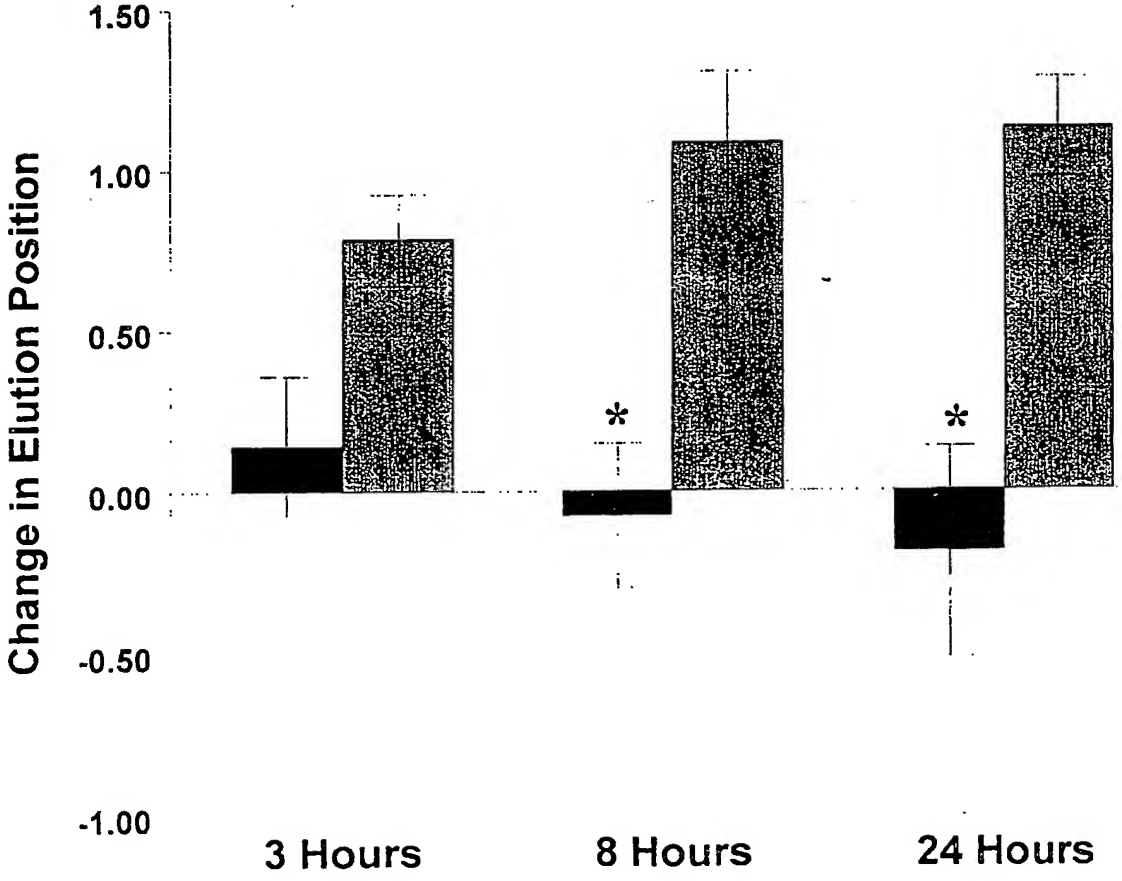
1.50
1.00
0.50
0.00
-0.50
-1.00
Change in Elution Position
*
*
3 Hours
8 Hours
24 Hours

FIG. 2A

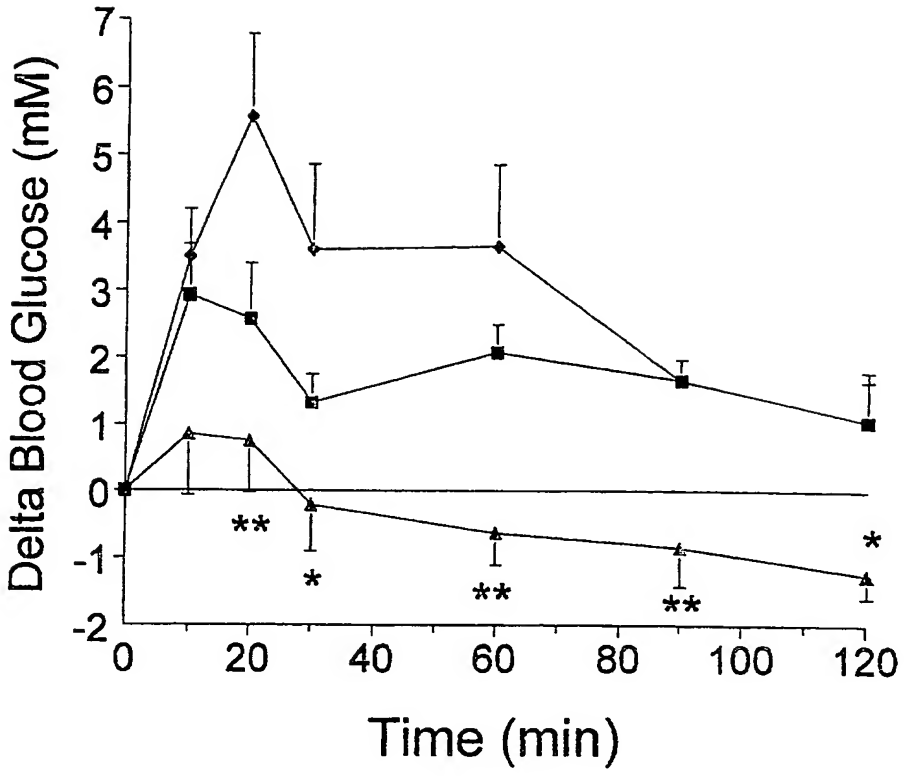
Delta Blood Glucose (mM)
7
6
5
4
3
2
1
0
-1
-2
0
20
40
60
80
100
120
Time (min)
**
*
**
**
*

FIG. 2B

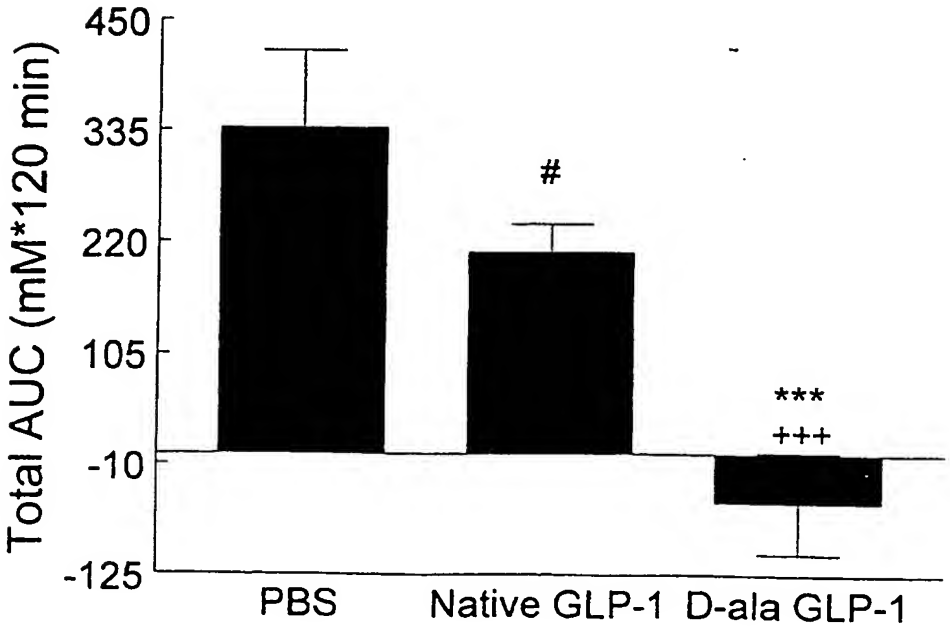
Total AUC (mM*120 min)
450
335
220
105
-10
-125
#
***
+++
PBS
Native GLP-1
D-ala GLP-1

FIGURE 3

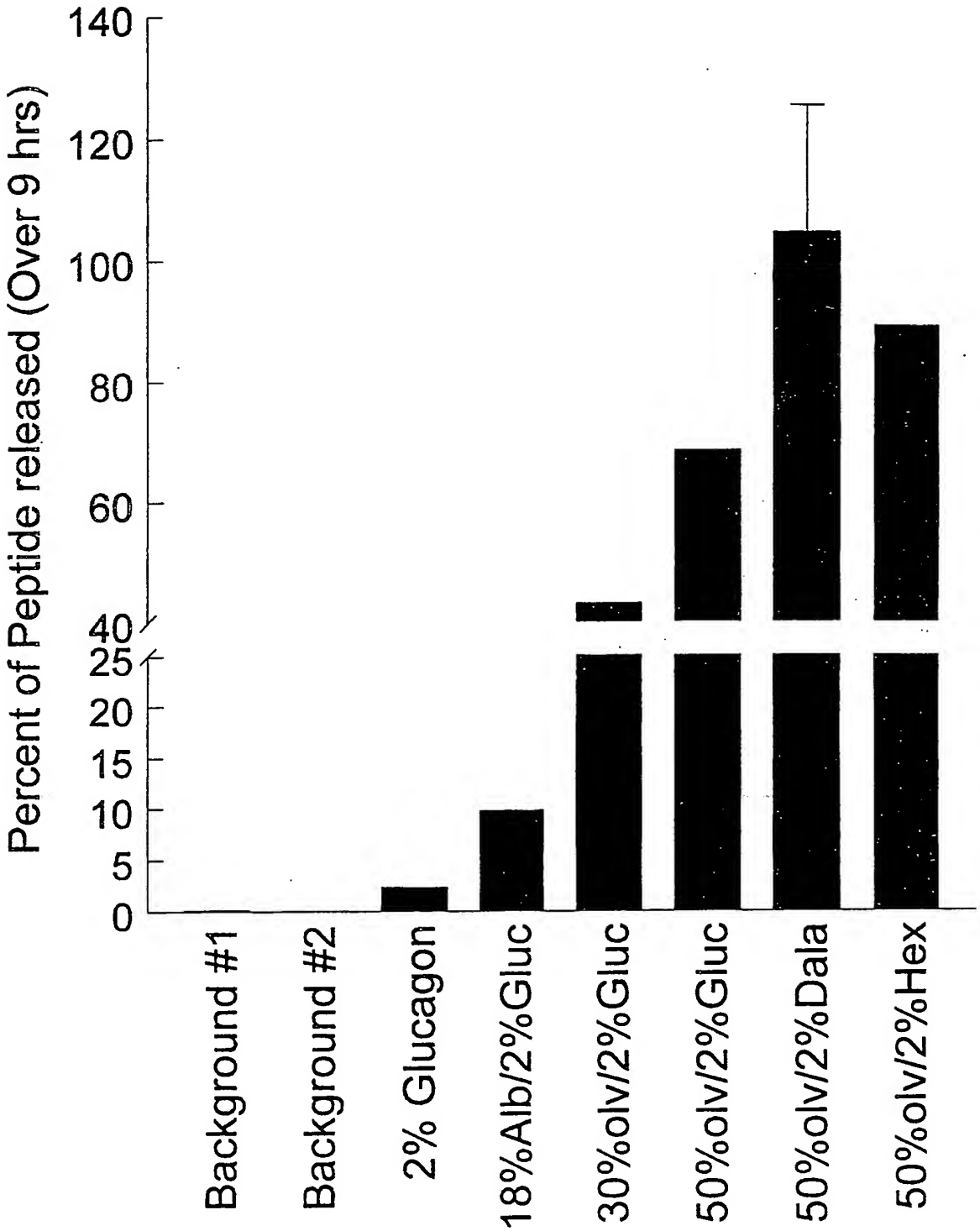
Percent of Peptide released (Over 9 hrs)
140
120
100
80
60
40
25
20
15
10
5
0
Background #1
Background #2
2% Glucagon
18%Alb/2%Gluc
30%olv/2%Gluc
50%olv/2%Gluc
50%olv/2%Dala
50%olv/2%Hex

FIG. 4A

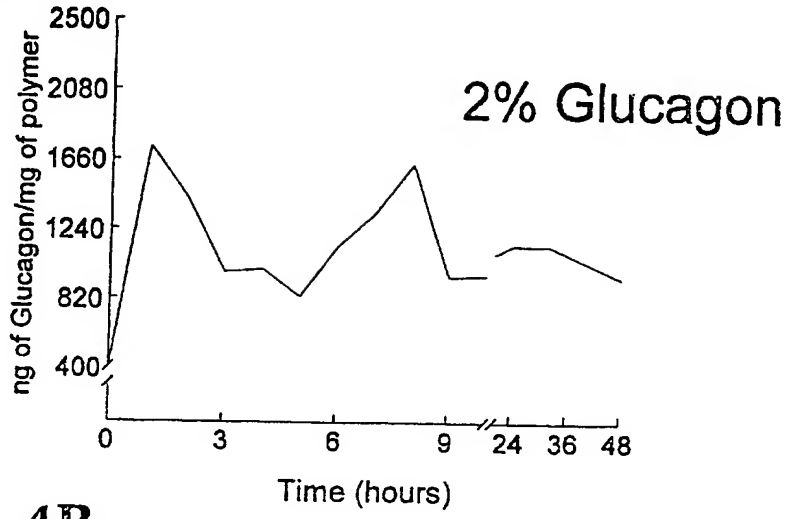

FIG. 4B

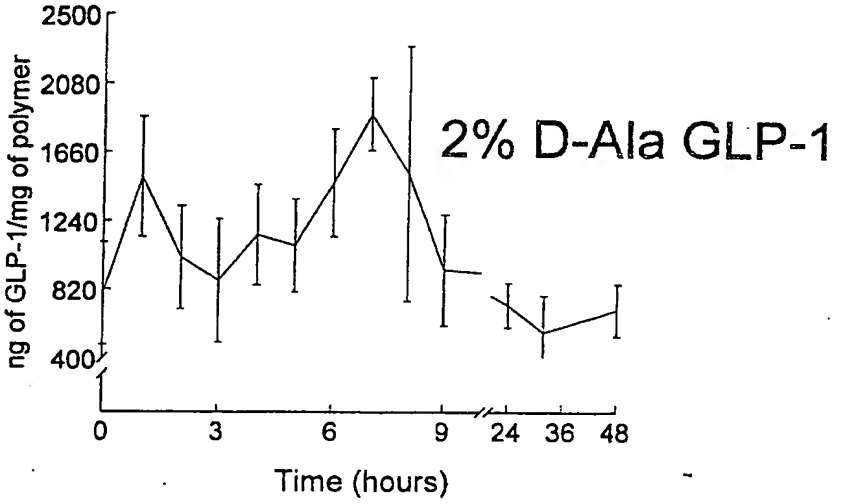

FIG. 4C

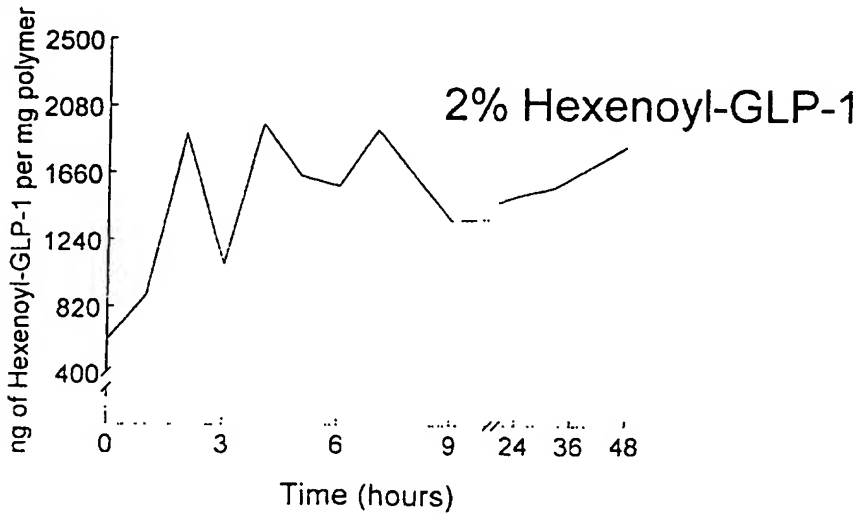

FIGURE 5

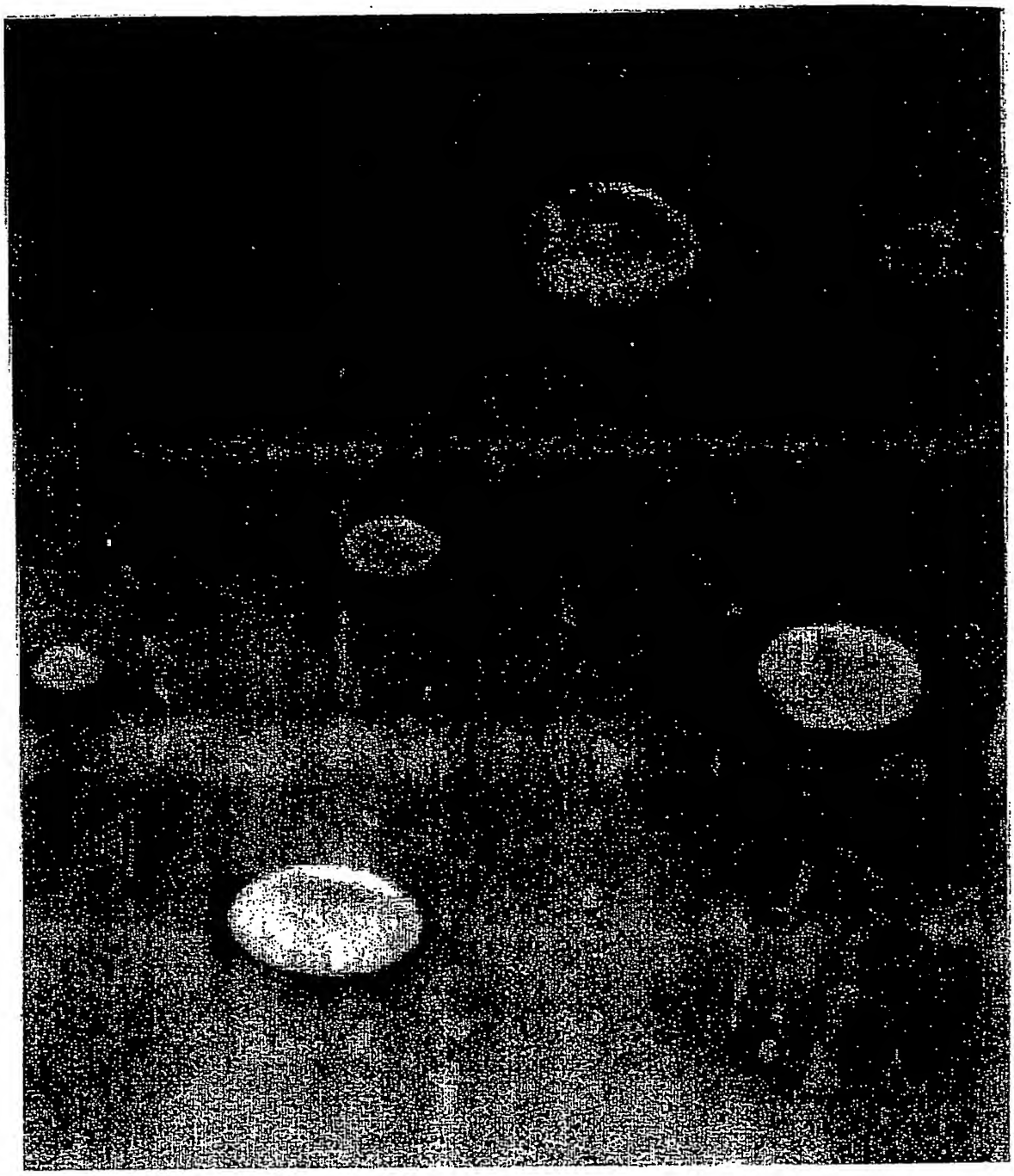

FIGURE 6

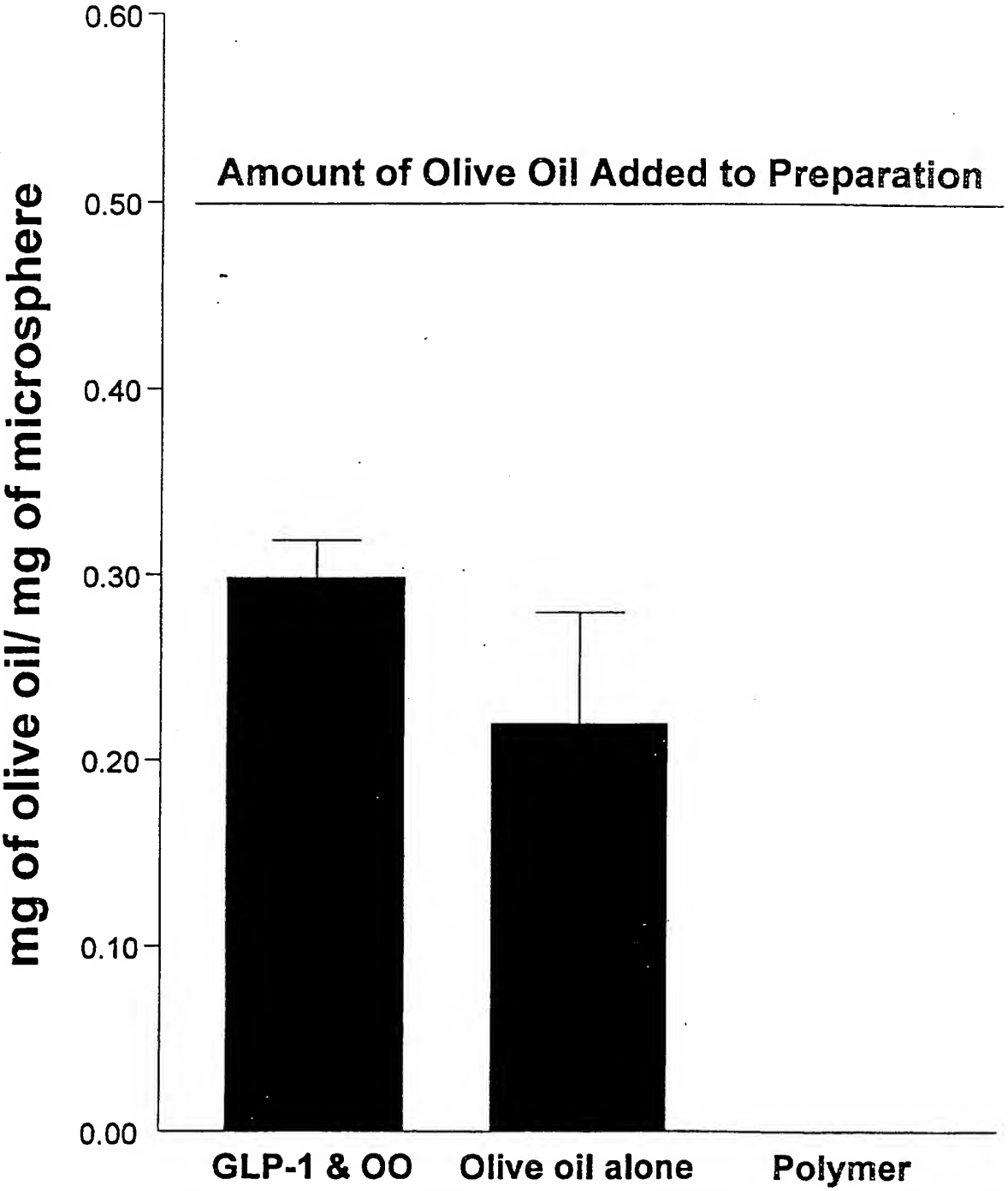
Amount of Olive Oil Added to Preparation
mg of olive oil/ mg of microsphere
0.60
0.50
0.40
0.30
0.20
0.10
0.00
GLP-1 & OO
Olive oil alone
Polymer

FIGURE 7

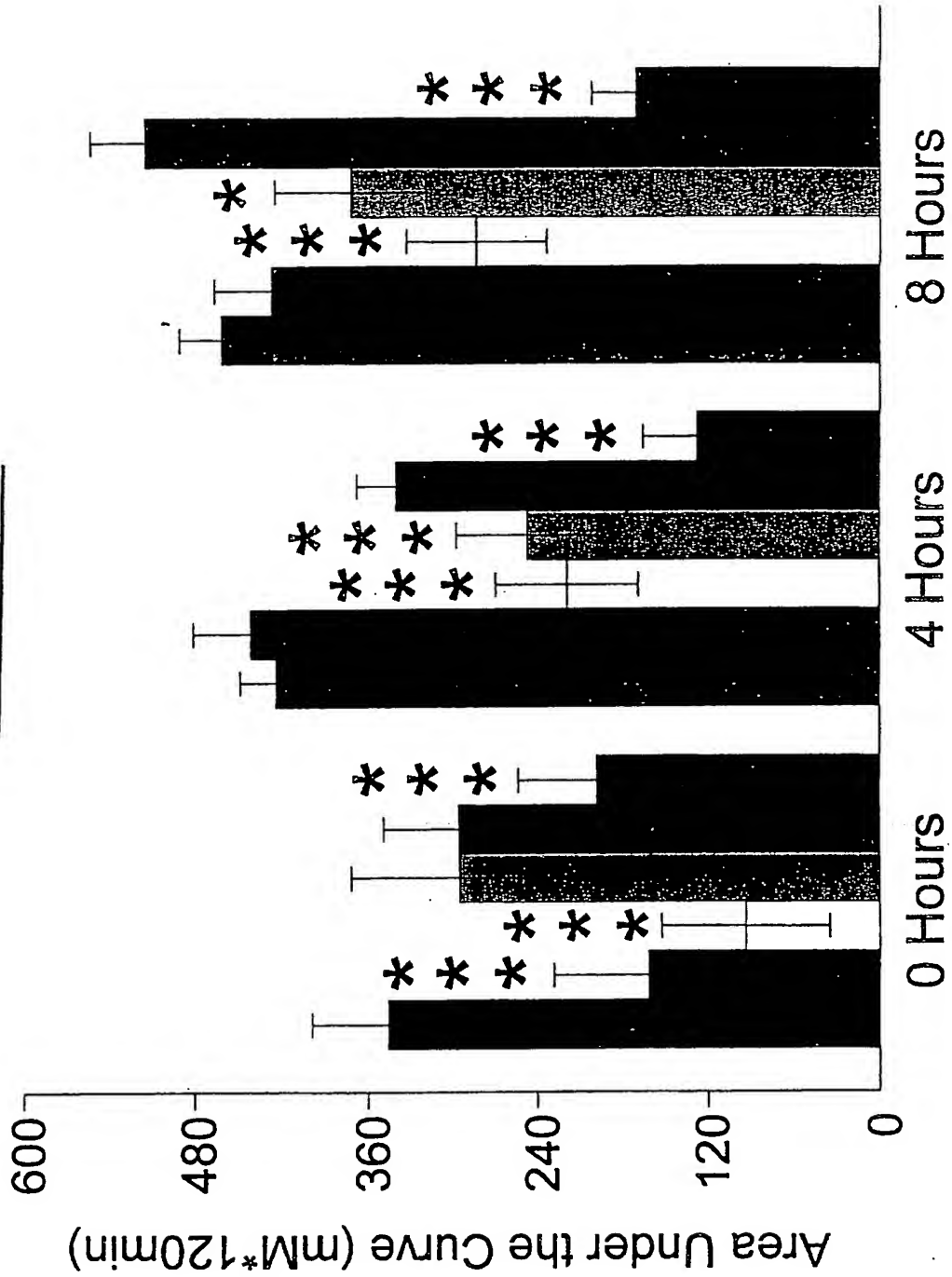
Area Under the Curve (mM*120min)
600
480
360
240
120
0
0 Hours
4 Hours
8 Hours
* * *
* * *
* * *
* * *
* * *
* * *
* * *
* * *
* * *

FIGURE 8

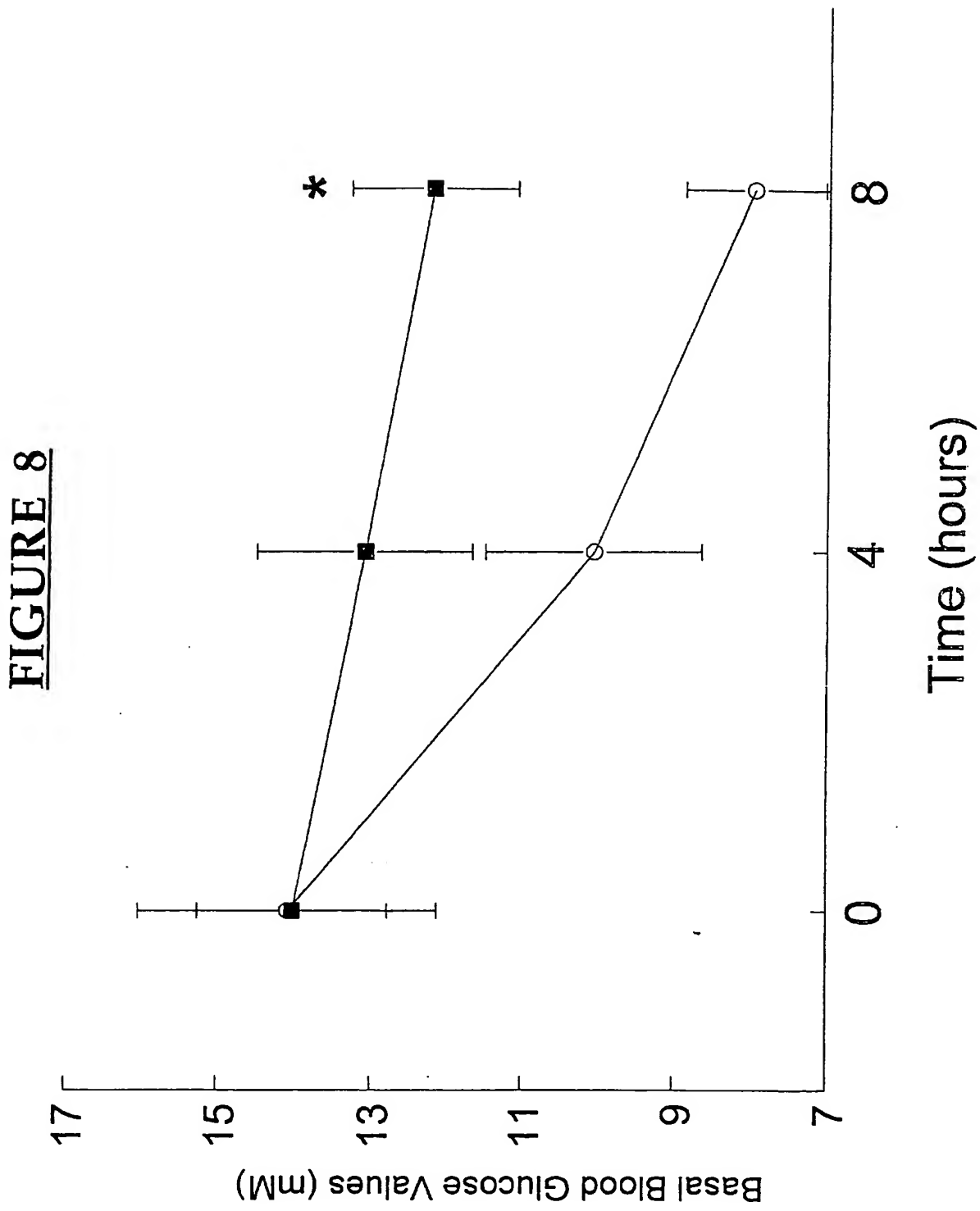

FIG. 9A

0 hr

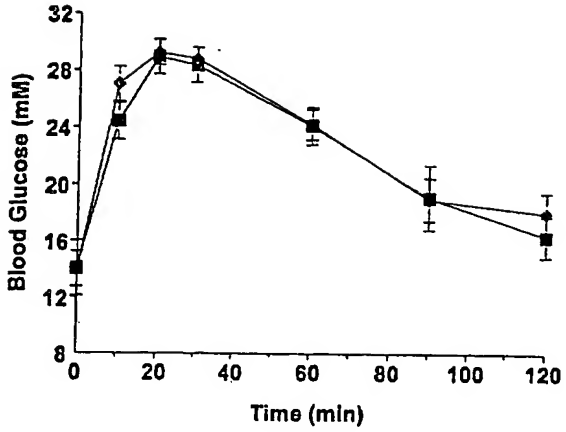

FIG. 9B

4 hr

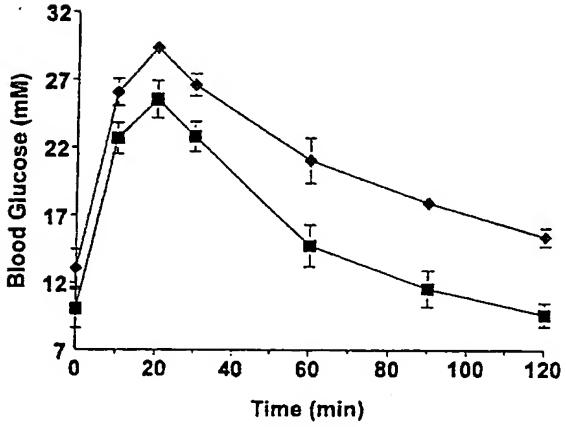

FIG. 9C

8 hr

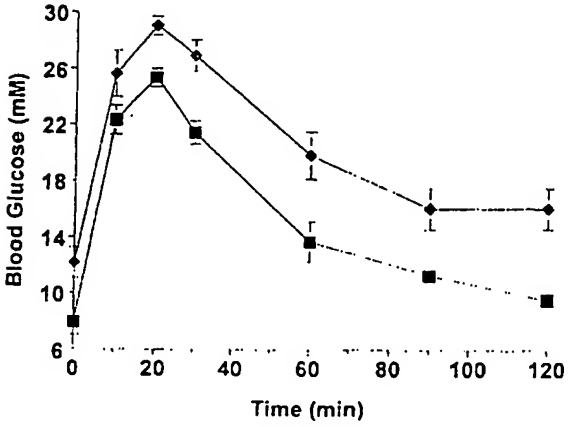

FIG. 10A

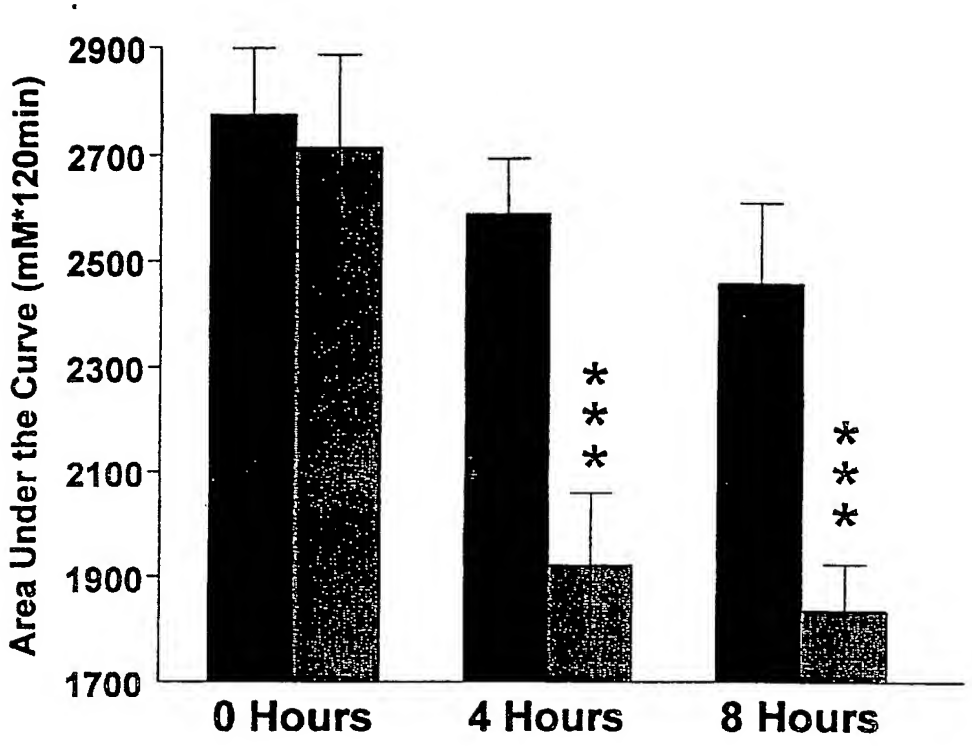

FIG. 10B

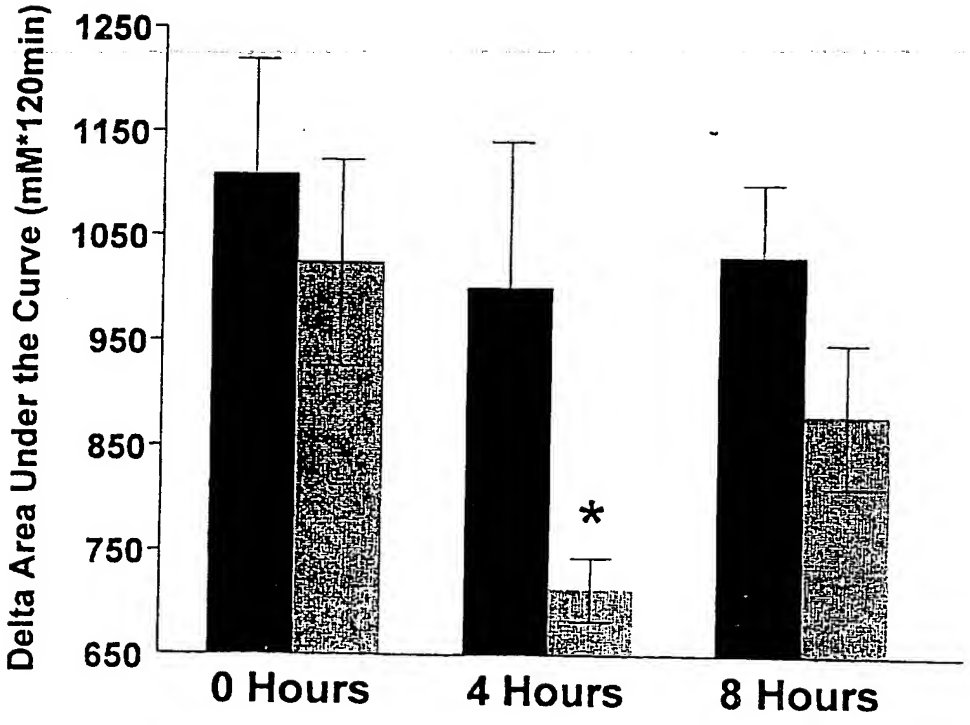

FIGURE 11